Attorney Docket No.: 4318.234-US

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of: Bisgaard-Frantzen et al. Confirmation No: 8501

Serial No.: 10/025,648 Group Art Unit: ~~1751~~

Filed: December 19, 2001 Examiner: Prouty

For: Amylase Variants

DECLARTION OF TORBEN V. BORCHERT UNDER 37 C.F.R. 1.132

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

I, Torben V. Borchert, do hereby declare as follows:

1. I am a Director at Novozymes in Bagsvaerd, Denmark, where I am responsible for research and development in protein design. I have been an employee at Novozymes since August 1993. I have held positions as research scientist, senior scientist, manager and director and have been involved in and directed work on improving the properties of industrial enzymes by protein engineering techniques for the complete period. Various enzymes have been addressed including alpha-amylases. Details of my education, professional experience and publications are included in the copy of my *Curriculum Vitae* which is attached as an Appendix hereto.

2. I am an inventor of the subject matter claimed in U.S. Application Serial No. 10/025,648. I am familiar with Suzuki et al.. J. Bio. Chem, Vol. 264, No. 32, 18933-18938 (1989) and Bisgaard-Frantzen et a. WO 95/10603.

3. Under my supervision, an experiment was carried out comparing the deletion of R179-G180 in *Bacillus stearothermophilus* alpha-amylase (BSG) to the deletion of R176-G177 in *Bacillus amyloliquefaciens* alpha-amyase (BAN), as described below.

4. A BSG variant having the deletion of R179-G180 (BSGdel) and a BAN variant having the deletion R176-G177 (BANdel) were constructed by standard mutagenesis methods and the sequences verified by DNA sequencing. Other than the deletion of R179-G180 in BSG and the deletion of R176-G177 in BAN, the variants were otherwise identical to the wild type alpha-amylases.

5. *Bacillus subtilis* strains expressing the wild type enzyme or variant enzyme, respectively for BAN and BSG, were grown under identical conditions in PS-1 media in shakeflasks for 4 days at 37 °C at 275 RPM, and were harvested by centrifugation of the samples for 5 minutes at 20,000 RMP, thus separating the cell pellet and the supernatant. The amylase containing supernatants were tested for residual activity after thermal inactivation carried out at 80 degree Celsius in a Britton Robinson (B-R) Buffer, pH: 5.9, and half life was calculated. The temperature of 80 degree Celsius was chosen as the highest temperature where both BAN and BSG wild type and derived variants could be reliably compared. The supernatants were diluted in B-R buffer to a suitable activity level and were aliquoted in 8 portions of 100 µl each. These samples were heat-treated in a PCR machine for the indicated times and at the indicated temperatures. The heat treatment was stopped by transferring the samples to ice and the samples were left there until activity was measured and residual activity calculated.

Experimental protocol:

1) Centrifuged the sample for 5 minutes at 20.000 rpm. Use the supernatant.
2) Dilute the sample in BR buffer pH 5.9 in order to achieve an activity level that will result in an OD650 of approximately 1.0 in the un-heated sample.
3) Substrate : Suspend 1 Phadebas amylase test tablet from Pharmacia in 5 ml B-R buffer pH 5.9.
4) 575 µl substrate in 1.5 ml eppendorftube is preheated 5 min. at 37 °C with shaking.
5) Add 25 µl sample at time 0 and continue shaking.
6) Add 100µl 1 M NaOH at time 15 min to stop the reaction.
7) Centrifuge the samples 5 min 20.000rpm.
8) Pipet 200 µl supernatant in a microtiterplate
9) Measure end-point at 650 nm.

Buffers:

B-R (Britton Robinson) buffer pH 5.9

50 mM acetic acid, 50 mM boric acid, 50 mM phosphoric acid,0.1 mM calcium chloride and 0.01% BRIJ 35 are mixed and pH is adjusted to 5.9 with NaOH.

6. Thermal inactivation trials. For BAN wild type and BAN variant one experiment was carried out with double determination for each time point. For BSG wild type and BSG variant two series of experiments were carried out due to the necessary, long incubation times.

Residual activity data (100% equals zero time) from the experiment is shown below.

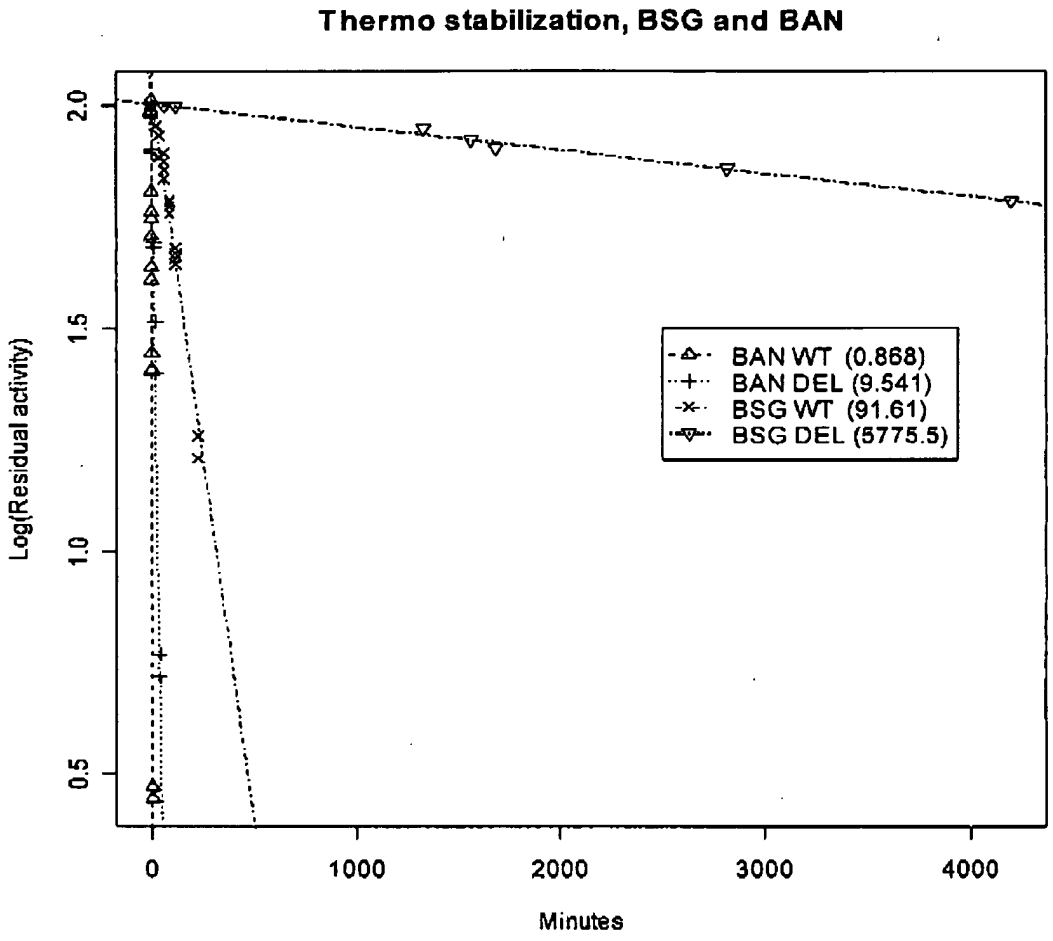
BAN Wild type:			BANdel			BSG Wild type				BSGdel		
	Min	Res act		Min	Res act		Min	Res act	exp		min res act	exp
1	0.0	102.697998	1	0	100.108814	1	0	99.19817	1	1	0 100	1
2	0.0	97.302002	2	0	99.891186	2	0	100.80183	1	2	65 100	1
3	0.5	96.083551	3	5	78.781284	3	20	89.57617	1	3	120 100	1
4	0.5	97.127937	4	5	80.087051	4	20	90.49255	1	4	1320 89	1
5	1.0	57.789382	5	10	48.095756	5	40	77.09049	1	5	1560 84	2
6	1.0	64.055701	6	10	49.183896	6	40	76.51775	1	6	1680 80	1
7	1.5	55.526545	7	20	25.244831	7	60	68.72852	1	7	2820 72	2
8	1.5	50.826806	8	20	32.861806	8	60	71.82131	1	9	4200 61	2
9	2.0	40.557006	9	40	5.223069	9	90	60.71019	1			
10	2.0	43.342037	10	40	5.875952	10	90	61.28293	1			
11	3.0	25.587467				11	120	44.10080	1			
12	3.0	27.850305				12	120	47.99542	1			
13	4.0	2.959095				13	0	99.37947	2			
14	4.0	2.785030				14	0	100.62053	2			
						15	20	90.31026	2			
						16	40	85.91885	2			
						17	65	74.84487	2			
						18	65	78.37709	2			

		19 90 57.37470 2 20 90 59.37947 2 21 120 46.58711 2 22 120 45.53699 2 23 225 16.22912 2 24 225 18.23389 2	

7. For each data series, a regression line was computed and the half-life was computed based on the regression line. The two data-series for BSG wild type gave different slopes (p=0.01), so they were treated both separately and as one series. The two data series for BSGdel give consistent slopes (p=0.96), so they are treated as one series. In the table, the half-lives are compared and the improvement factors are compared. The numbers in parenthesis corresponds to the two data series on BSG wild type treated separately.

	Half-life @ 80 degree Celcius	Improvement	Relative improvement
BAN WT	0.9 min		
BANdel	9.5 min	11x	
BSG WT	92 min (87-111)		
BSGdel	5775 min	63x (52x – 66x)	5.7x (4.7x – 6x)

8. A graphical illustration is provided below.



9. The deletion of R179-G180 in BSG has a pronounced and very surprising effect on the thermal stability in BSG as compared to the deletion of R176-G177 in BAN. The deletion of R179-G180 in BSG causes a 63 fold increase in half-life at 80 degree Celsius whereas the deletion of R176-G177 in BAN causes only an 11 fold increase in half-life at the same conditions. The deletion of R179-G180 in BSG gives a relative improvement of thermal stability which is 5 to 6 times higher than what is seen in BAN having the deletion of R176-G177. These results are statistically significant and very surprising as the effect of the double deletion in BSG is significantly greater than what would have been expected based on the

combined teachings of Suzuki et al. (JBC 260:6518, 1989) in view of Bisgaard-Frantzen et al., WO 95/10603. The statistical analysis is attached as Appendix 1.

10. All statements made herein of my own knowledge are true and all statements made herein on information and belief are believed true. Further, I am aware that willful false statements and the like are punishable by fine, imprisonment, or both, 18 U.S.C. § 1001, and that such willful false statements may jeopardize the validity of the involved Svendsen application, as well as the position of Novozymes in the above-captioned interference.

Date SEP 6th, 2004

Torben V. Borchert, Ph.D.